SuperArray Bioscience Corporation

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)

biomol

User Manual

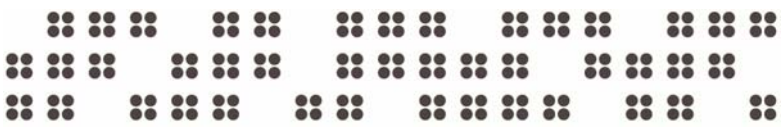
Part # 1017A

Version 1.5 4/21/2006

RT^2*Profiler*™ PCR Array

PCR-BASED PATHWAY-FOCUSED
GENE EXPRESSION PROFILING IN A 96-WELL FORMAT

See Purchaser Notification for limited use license and warranty information (page 3).



RT²*Profiler*™ PCR Array
For Catalog Numbers Prefixes: APH, APM, and APR

PCR-Based Pathway-Focused
Gene Expression Profiling in a 96-Well Format

USER MANUAL

ORDERING INFORMATION AND TECHNICAL SERVICE

- TEL: 0800-2466651 (D); +49-8532600 (outside D)
- FAX: 0800-2466652 (D); +49-85326022 (outside D)
- ON-LINE ORDER: www.biomol.de
- E-MAIL: info@biomol.de (to place an order)
  ts@biomol.de (for technical support)

You may place orders by phone, fax, e-mail or from our website. Each order should include the following information:

- Your contact information (name, phone, email address)
- Product name, catalog number and quantity
- Purchase order number or credit card information (Visa or MasterCard)
- Shipping address
- Billing address

For more information, visit us at http://www.superarray.com or http://www.biomol.de

SuperArray Bioscience Corp.
7320 Executive Way, Suite 101
Frederick, MD 21704
USA

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LIMITED PRODUCT WARRANTY

This product is intended for research purposes only and is not intended for drug or diagnostic purposes or for human use. This warranty limits our liability to replace this product in the event the product fails to perform due to any manufacturing defect. SuperArray Bioscience Corporation makes no other warranties of any kind, expressed or implied, including without limitation, warranties of merchantability or fitness for a particular purpose. SuperArray Bioscience Corporation shall not be liable for any direct, indirect, consequential or incidental damages arising out of the use, the results of use or the inability to use this product.

NOTICE TO PURCHASER

The purchase of RT²*Profiler*™ PCR Array includes a limited, nonexclusive license to use the kit components for research use only. This license does not grant rights to use the kit components for reproduction of any primer pair mix, to modify kit components for resale or to use RT²*Profiler*™ PCR Array to manufacture commercial products without written approval of SuperArray Bioscience Corporation. No other license, expressed, implied or by estoppels, is granted. U.S. patents may cover certain isolated DNA sequences included in the RT²*Profiler*™PCR Array. Presently, it is not clear under U.S. laws whether commercial users must obtain licenses from the owners of the rights to these U.S. patents before using RT²*Profiler*™ PCR Array.

I. BACKGROUND AND INTRODUCTION

Real-time reverse transcription (RT) PCR is the most sensitive and reliable method for gene expression analysis. Its wide dynamic range makes real-time RT-PCR the preferred choice for the simultaneous quantification of both rare and abundant genes in the same sample. The RT²*Profiler* PCR Array takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of many genes simultaneously. RT²*Profiler* PCR Arrays are designed to analyze a panel of genes related to a disease state or biological pathway. The product is especially suitable for researchers who are more familiar with or prefer real-time PCR technology but are looking for the multi-gene profiling capabilities of a microarray.

To complete the PCR Array procedure, start by converting your experimental RNA samples into first strand cDNA, the template for the polymerase chain reaction, using our ReactionReady™ First Strand cDNA Synthesis Kit. (See Figure 1 for an overview of the PCR Array procedure.) Then, mix your template with one of our instrument-specific and ready-to-use PCR master mixes. Aliquot the mixture into each well of the same plate containing pre-dispensed gene-specific primer sets. Perform PCR, and finally, determine relative expression with your real-time instrument and the $\Delta\Delta C_t$ method.

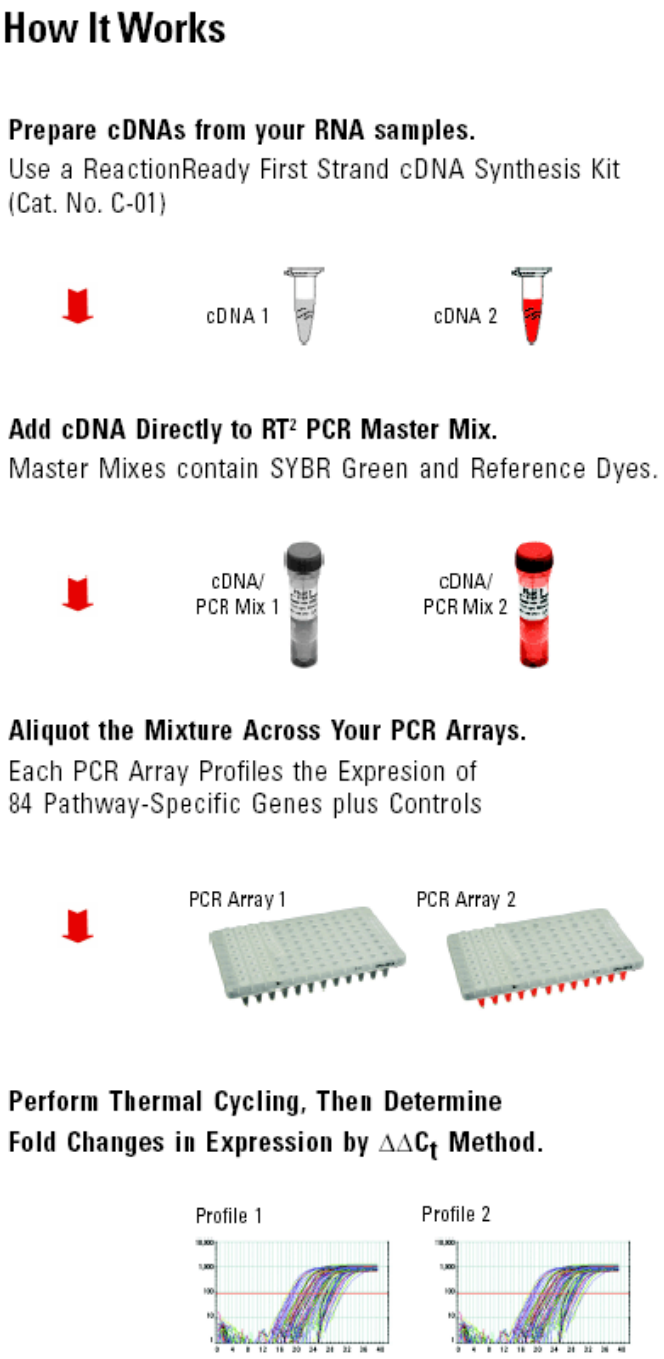
Each array is a 96-well plate that includes primer sets for a thoroughly researched set of 84 relevant, pathway- or disease-focused genes plus five housekeeping genes and two negative controls. (See Figure 2 for the layout of a typical PCR Array.) These primer sets and our master mixes have both been optimized hand-in-hand for SYBR Green real-time detection, providing the PCR Arrays with superior sensitivity and wide linear dynamic ranges. The simplicity of the PCR Arrays also makes them accessible for routine use in every research laboratory.

Benefits of the RT²*Profiler*™ PCR Arrays:

Pathway Focused:
Profile the expression of a panel of genes relevant to a pathway or disease state.
Simple and Accurate:
Simple real-time PCR procedure provides high sensitivity and wide dynamic range.
Designed for Routine Use:
Bring expression profiling to almost any lab with a real-time PCR instrument.

Combine microarray profiling capabilities with real-time PCR performance!

Figure 1: Overview of the PCR Array procedure.


How It Works
Prepare cDNAs from your RNA samples.
Use a ReactionReady First Strand cDNA Synthesis Kit (Cat. No. C-01)
cDNA 1
cDNA 2
Add cDNA Directly to RT² PCR Master Mix.
Master Mixes contain SYBR Green and Reference Dyes.
cDNA/ PCR Mix 1
cDNA/ PCR Mix 2
Aliquot the Mixture Across Your PCR Arrays.
Each PCR Array Profiles the Expresion of 84 Pathway-Specific Genes plus Controls
PCR Array 1
PCR Array 2
Perform Thermal Cycling, Then Determine Fold Changes in Expression by ΔΔC$_t$ Method.
Profile 1
Profile 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
C	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36
D	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
E	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60
F	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
G	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84
H	HK1	HK2	HK3	HK4	HK5	HK5	HK5	HK5	HK5	HK5	NRT	NTC

Figure 2: Layout of the Cataloged PCR Arrays.

Wells A1 through G12 contain primers for genes from the same biological pathway (G1-G84). The product information included with each array contains a list of these genes. You will add aliquots of the same cDNA template to each of these wells.

Wells H1 through H5 contain a panel of housekeeping genes (HK1-HK5) used for normalizing the PCR Array data. Again, refer to your array's product information for the list of the housekeeping genes. You will also add aliquots of the same cDNA template used for wells A1 through G12 to each of these wells.

Wells H6 through H10 contain the same housekeeping gene primers as well H5 (HK5). These wells are designed to estimate the linear dynamic range of the assay. You will add a different 10-fold serial dilution the cDNA template to each of these wells.

Well H11 also contains the same housekeeping gene primers as well H5 (HK5). This reaction will contain your RNA diluted into master mix, and will serve as the no reverse transcription control (NRT) to estimate the level of genomic DNA contamination in the RNA sample.

Well H12 also contains the same housekeeping gene primers as well H5 (HK5). This reaction will contain only master mix, and will serve as the no PCR template control (NTC) or water control to estimate the level of overall DNA contamination in the PCR system.

Custom PCR Arrays have your specified layout, and the product information enclosed with the array specifies the layout and the genes included.

II. Materials Provided:

Each PCR Array includes the array itself and either twelve (12) optical thin-wall 8-cap strips (A and B formats) or one (1) optical adhesive film per array (C format).
PCR Arrays are shipped in sets of two or twelve (2 or 12) and come in three different plate formats, each tailored to a specific subset of real-time PCR instruments:

Format:	For Real-Time Instruments:
A	ABI 7000, 7300, 7500 standard, 7700, 7900 standard Bio-Rad iCycler, MyiQ Bio-Rad (MJ Research) Chromo 4
B	Stratagene Mx3005p, Mx3000p, Mx4000
C	ABI 7500 FAST, 7900 FAST
D	Bio-Rad (MJ Research) Opticon 2 and 4

The format of the PCR Array is indicated by the last digit of the catalog number.
NOTE: *Be sure that you have the correct PCR Array format for your instrument before starting the experiment.*

Storage Conditions:
All components included in this kit are shipped at ambient temperature but must be stored at -20 °C where they are guaranteed as is for 6 months from the date received.

III. Additional Materials:

A. RNA Isolation Kit: See Page 8 for specific suggestions.

B. ReactionReady™ First Strand cDNA Synthesis Kit (Cat. No. C-01)

C. SuperArray RT² Real-Time™ SYBR Green PCR Master Mix
MANDATORY for a Complete and Successful Experiment
Be sure to pick the correct one for the instrumentation in your laboratory.

RT² Real-Time™ SYBR Green / ROX (Cat. No. PA-012)
Specifically designed for all ABI and Stratagene Instrumentation
RT² Real-Time™ SYBR Green / Fluorescein (Cat. No. PA-011)
Specifically designed for BioRad iCylcer® and MyiQ®
RT² Real-Time™ SYBR Green (Cat. No. PA-010)
Specifically designed for instrumentation not requiring a reference dye

D. Equipment:
Real-time instrumentation (= thermal cycler with fluorescent detection)
For instrument recommendations, see the list of master mixes above.

NOTE: *The PCR Arrays are **NOT** recommended for the Cepheid SmartCycler® or the Roche LightCycler® 2.0 due to the different non-traditional hot block arrangements in those instruments.*

IV. Complementary Products: XpressRef™ Universal Total RNA:

Universal RNA to control PCR conditions is available from the following species:
Human XpressRef™ Universal Total RNA (Cat. No. GA-005)
Mouse XpressRef™ Universal Total RNA (Cat. No. GA-006)
Rat XpressRef™ Universal Total RNA (Cat. No. GA-007)

V. Protocol:

Please read through this entire protocol before beginning your experiment.
RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol.

NOTE: Master Mix Considerations

The use of SuperArray's RT² Real-Time™ SYBR Green PCR master mixes is absolutely essential for obtaining accurate results with the RT²*Profiler* PCR Arrays. The chemically-modified and tightly controlled HotStart enzyme in these master mixes uniquely provides more accurate SYBR Green results by preventing the amplification of primer dimers and other non-specific products. The RT² Real-Time™ PCR Master Mix formulations also include other proprietary chemical components that significantly minimize primer dimer formation ensuring high amplification efficiencies for even the most difficult to amplify genes.

The performance of our RT²*Profiler*™ PCR Arrays is only guaranteed with our RT² Real-Time™ SYBR Green PCR master mixes and not any other source of master mix. In fact, when we test other sources of master mix with our primer sets, we frequently see primer dimers and other non-specific products that confound SYBR-Green based real-time PCR detection. Because each instrument uses a different reference dye to normalize their optics, be sure that you are using the correct master mix for the instrumentation in your laboratory.

NOTE: Preparing a Workspace Free of DNA Contamination

For accurate and reproducible PCR Array results, it is very important to avoid contamination of the assay with foreign DNA. Any DNA contamination will artificially inflate the SYBR Green signal yielding skewed gene expression profiles and false positive signals. The most common sources of DNA contamination are the products of previous experiments spread into the air of your working environment. Please follow the recommendations below on how to set up and maintain a working environment free of DNA contamination.

1. Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (H_20) and lab ware (tips and tubes).
2. Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate your PCR workspace and lab ware (pipettor barrels, tube racks, etc.) before each new use with UV light to render any contaminating DNA ineffective in PCR through the formation of thymidine dimers or with 10% bleach to chemically inactivate and degrade any DNA.
3. Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any lab ware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.
4. Do not peel the protective film from the PCR Array plate until immediately ready to use. Do not leave lab ware (tubes and tip boxes) exposed to the air for long periods of time.

A. RNA Preparation and Quality Control:

High quality RNA is ESSENTIAL for obtaining good real-time PCR results.

The most important prerequisite for any gene expression analysis experiment is consistent, high-quality RNA from every experimental sample. Therefore, the sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants will either degrade the RNA or decrease the efficiency of (if not block completely) the enzyme activities necessary for optimal reverse transcription and real-time PCR performance.

1. Recommended RNA Preparation Methods:

High quality total RNA for your real-time PCR experiment must be prepared using one of the following methods, each specific for your biological sample:

Cultured Cells: Use the Qiagen RNeasy® Mini Kit (Catalog # 74103). Be sure to include the recommended DNase treatment step.

Tissue Samples: Use a two-step protocol. First, extract RNA from the tissue using the TRIzol® protocol (Invitrogen, Catalog # 15596-026). Be sure to use a sufficient amount of TRIzol® reagent. During homogenization, add a volume of reagent at least ten times greater than the tissue volume. Then after the ethanol precipitation step, further clean up the RNA using the Qiagen RNeasy® Mini Kit (Catalog # 74103). Be sure to include the recommended DNase treatment step.

Whole Blood Samples: Before RNA preparation, red blood cells (RBC) must be removed from whole blood samples using a density gradient centrifugation medium (for example, Lymphoprep, Greiner Bio-One, Catalog # 1031966). The white blood cell fraction is then used for RNA isolation with the Qiagen RNeasy® Mini Kit (Catalog # 74103). Be sure to include the recommended DNase treatment step. Alternatively, the PAXgene Blood RNA Kit (Qiagen, Catalog # 762134) can also be used to prepare total RNA from whole blood samples.

Total RNA Isolated Using a Phenol-Based Method: If you have already prepared total RNA from any biological source material using a phenol-based method (such as TRIzol, RNAzol, etc.), you must clean up the RNA with the Qiagen RNeasy® Mini Kit (Catalog # 74103) to insure optimal performance. Be sure to include the recommended DNase treatment step.

For Other Biological Samples: Refer to existing literature to find isolation protocols for high-quality RNA from other biological samples or contact a Technical Support representative.

For best results from the PCR Array, all RNA samples should be suspended in the RNase-free water provided with the RNA Isolation kit (not DEPC-treated water), or alternatively in RNase-free 10 mM Tris buffer pH 8.0.

2. RNA Quality Control:

For best results from the PCR Array, all RNA samples should also demonstrate consistent quality according to the following criteria:

a. RNA Concentration and Purity by UV Spectrophotometry

NOTE: *Prepare dilutions and measure absorbance in an RNase-free 10 mM Tris, pH 8.0 buffer. The spectral properties of nucleic acids are highly dependent on pH.*

i) Concentration by A_{260} should be greater than 4 μg / ml total RNA
ii) A_{260}:A_{280} ratio should be greater than 2.0.
iii) A_{260}:A_{230} ratio should be greater than 1.7.

b. Ribosomal RNA band integrity

Electrophorese a fraction of each RNA sample on a denaturing agarose gel or on an Agilent BioAnalyzer® using an RNA 6000 Nano LabChip® and verify that there is a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) bands or peaks. Any smearing or shoulder to the rRNA bands or peaks indicates that degradation has occurred in the RNA sample.

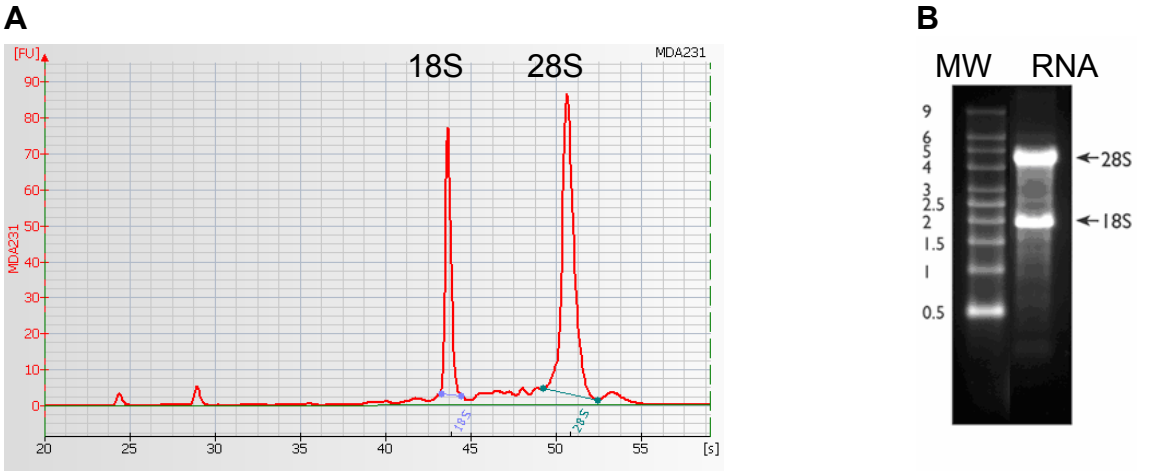


Figure 3: Good Ribosomal RNA Band Integrity Is Important for Best Results from the PCR Array. Panel **A** displays an Agilent BioAnalyzer® electropherogram of a high-quality total RNA preparation showing sharp peaks without shoulders (especially to the left of each peak) for the 18S and 28S ribosomal RNA (left to right). Panel **B**, right-hand lane, displays an analysis of the same high-quality total RNA preparation by agarose gel electrophoresis demonstrating sharp bands (especially at the bottom of each band) for the 28S and 18S ribosomal RNA (top to bottom).

Because some contaminants are difficult to detect by simply looking at RNA integrity and can be missed by UV spectrophotometry, it is essential to choose the proper RNA isolation method for your biological sample as described above.

3. Genomic DNA Contamination:

Minimizing or eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using the PCR Array. The "no reverse transcription" (NRT) control in well H11 specifically tests for genomic DNA contamination and should yield real-time C_t values greater than 35. If the C_t values are less than 35, then genomic contamination is apparent.

While isolating your total RNA, be sure to include any DNase treatment steps in the recommended RNA isolation procedure. You may also re-treat your RNA sample after isolation with a good source of RNase-free DNase followed by re-purification using a spin-column based method (e.g., the Qiagen RNeasy Mini Kit). Increase the number of units of enzyme and incubation time beyond the recommendations made by the original manufacturer for the RNase-free DNase.

4. Amount Considerations:

The PCR Array will yield relative gene expression profiles with as little as 50 ng or as much as 5 μg total RNA per array. However, the use of smaller amounts of RNA will likely not detect genes expressed at a low level. In other words, the optimal amount of starting material depends upon the relative abundance of the transcripts of interest. Lower abundance transcripts require more RNA; higher abundance transcripts require less RNA. Greater amounts of input total RNA yield a greater number of positive calls; that is, genes expressed in the linear dynamic range of the method. Lower amounts of input total RNA yield a smaller number of positive calls.

For successful results, we recommend starting with as much input material as possible, for example, 0.5 or 1.0 μg of total RNA.

B. First Strand cDNA Synthesis
Using SuperArray Catalog Number C-01, sold separately

NOTE: *The buffer components and the magnesium concentration of the reverse transcription reaction using this kit are more compatible with our PCR master mixes than other sources of first strand cDNA synthesis kits or reverse transcriptases.*

1. Prepare the Annealing Mixture:

For each RNA sample, combine the following in a sterile PCR tube:

Total RNA	0.05 to 5.0	µg *
Buffer **P**	1.0	µl
RNase-free **H_2O** to a final volume of	10.0	µl

Use the same amount of total RNA in this reaction for every sample.

Mix the contents gently with a pipettor followed by brief centrifugation. Place the mixture in a thermal cycler at 70 °C for 3 min. Cool to 37 °C and incubate there for 10 min.

NOTE: *First time users are recommended to start with 0.5 or 1.0 µg of total RNA.*

NOTE: *Be sure to save at least 1 µl of each RNA preparation to set up your no reverse transcription (NRT) controls in well H11 of each PCR Array.*

2. Prepare the RT Cocktail:

This mixture can be prepared while the Annealing Mixture is incubating at 37 °C.

RT Cocktail	1 reaction	2 reactions	4 reactions
Buffer **BC** (5X RT Buffer)	4 µl	8 µl	16 µl
RNase-free **H_2O**	4 µl	8 µl	16 µl
RI (RNase Inhibitor)	1 µl	2 µl	4 µl
RE (Reverse Transcriptase)	1 µl	2 µl	4 µl
Final Volume	10 µl	20 µl	40 µl

Warm the RT Cocktail at 37 °C for 1 min before proceeding to the next step.

3. First Strand cDNA Synthesis Reaction:

Add 10 µl of RT Cocktail to each 10 µl-Annealing Mixture. Mix well but gently with a pipettor and continue incubation at 37 °C for 60 min. Heat at 95 °C for 5 min to degrade the RNA and to inactivate the reverse transcriptase.

Add 80 µl of ddH_2O to each 20-µl of cDNA synthesis reaction. Mix well.

Hold the finished First Strand cDNA Synthesis Reaction on ice until the next step or store overnight at -20 °C.

C. Performing Real-Time PCR:

NOTE: *The use of SuperArray's RT² Real-Time™ SYBR Green PCR Master Mixes is absolutely critical for obtaining accurate results from the PCR Array. Be sure to use the correct master mix for your instrument before continuing with this protocol (See Pages 7 and 8).*

NOTE: *The use of the correct PCR Array plate format is also critical to the success of this experiment. Be sure that you have the correct PCR Array format for your instrument before continuing with this protocol (See Page 7).*

NOTE: *Accurate pipetting is very critical for the success of this protocol. Be sure that all of your micro-pipettors are calibrated before beginning this procedure. Also, be sure to not introduce any bubbles into the wells of the PCR Array.*

1. Sample Preparation:

   a. For wells A1 through H5:

Mix the following components in a 5-ml tube or a multi-channel pipettor reservoir:	
2X SuperArray PCR master mix	1225 µl
Diluted first strand cDNA synthesis reaction	98 µl
ddH_2O	1127 µl
Total volume	2450 µl

   b. For wells H6 through H10:

In separate tubes, prepare five (5) 10-fold serial dilutions of the cocktail generated above for wells A1 – H5:					
For WELL	H6	H7	H8	H9	H10
1X PCR master mix[1]	27 µl	27 µl	27 µl	27 µl	27 µl
Material used for previous well[2]	3 µl	3 µl	3 µl	3 µl	3 µl

   c. In another separate tube, mix 1 µl of a 1:100 dilution of your original input total RNA with 24 µl of 1X PCR master mix[1] to use as the no reverse transcription (NRT) control in well H11.

   d. In another separate tube, prepare a 25-µl aliquot of 1X PCR master mix[1] to use as the no template control (NTC) in well H12.

[1]To prepare 1X PCR master mix, combine 100 µl each 2X SuperArray PCR master mix and ddH_2O.

[2]More specifically, mix 3 µl of the cocktail generated for wells A1 through H5 with 27 µl of 1X PCR master mix. Generate four more 10-fold serial dilutions of this mixture into 1X PCR master mix.

2. Adding samples to PCR Array:

   a. **CAREFULLY** remove the plate seal from the PCR Array.

   b. Add 25 µl of the appropriate cocktail to each well of the PCR array.
   Change the pipet tips each time to avoid cross-contaminating the reactions.

   c. **CAREFULLY** but tightly seal the PCR Array with the optical thin-wall 8-cap strips (Formats A, B, and D) or with the optical adhesive film (Format C).

NOTE: *Be sure that no bubbles appear in any of the wells of the PCR Array. To remove bubbles, tap the plate gently on the bench top or, if possible, centrifuge the plate briefly.*

   d. Place the plate on ice while setting up the appropriate PCR program below.

3. Performing Real-Time PCR Detection:

   a. Place one plate in your real-time thermal cycler.
   b. Enter and run the appropriate program for your real-time instrument:

For the ABI Instrumentation (7000, 7300, 7500, and 7900):

Cycles	Duration	Temperature
1	10 minutes[1]	95 °C
40	15 seconds	95 °C
	1 minute[2]	60 °C

For the BioRad iCycler® and all other instrumentation:

Cycles	Duration	Temperature
1	10 minutes[1]	95 °C
40	15 seconds	95 °C
	30 to 40 seconds[2,3]	55 °C
	30 seconds	72 °C

[1]The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.
[2]Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.
[3]Different instruments need different lengths of time to detect the fluorescent signal. Choose the annealing step (55 °C) time appropriate for your instrument.

   c. Calculate the threshold cycle (C_t) for each well using the instrument's software.

NOTE: *Be sure to follow the manufacturer's instructions for the proper operation and maintenance of your real-time instrument.*

4. Optional Quality Control:

   a. Dissociation (Melting) Curve:

Run a melting curve program immediately after the above PCR program, and generate a first derivative dissociation curve for each well in the entire plate using your instrument's software. No more than one peak should appear in each reaction at temperatures greater than 80 °C. If your instrument does not have a default melting curve program, run the following program instead:

95 °C, 1 min; 65 °C, 2 min (OPTICS OFF); 65 °C to 95 °C at 2 °C / sec (OPTICS ON).

If you decide not to obtain the dissociation curve immediately, save the plates at -20 °C as is, in case you need to perform this operation at a later point in time for troubleshooting purposes. When ready, simply warm the plate to room temperature, place it into your real-time instrument, and run the melting program described above.

NOTE: *Be sure to visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, make a note of which wells so that you may qualify your data analysis appropriately.*

   b. Agarose gel electrophoresis:

In either case, save the completed reactions at -20 °C in case agarose gel electrophoresis characterization is also needed for troubleshooting purposes. No more than one band should be apparent in each lane.

i. Mix 10 μl of each reaction with 2 μl of 6X agarose gel loading buffer.
ii. Load each sample into separate wells of a **2%** agarose gel containing 0.5 μg/ml ethidium bromide in 1X TAE.
iii. Load an appropriate amount of 100-bp DNA Step Ladder (Promega G695A) in an adjacent lane.
iv. Electrophorese in 1X TAE at 90V for 40 minutes or before the tracking dye runs off the gel.
v. Capture an image of the gel with a UV Trans Illuminator using a Gel-Doc Station (CCD camera) or high-speed film.

D. Data Analysis: $\Delta\Delta C_t$ Method

1. Examine the threshold cycle of the negative controls.

The No Template Control (NTC) tests for DNA contamination in your PCR system, while the No Reverse Transcription (NRT) control tests for contamination of the original RNA with genomic DNA. Both threshold cycles should be greater than 35. If the threshold cycle for either of these controls is less than 35, then the presence of DNA contamination is evident. See the Troubleshooting Guide for more information.

2. Change all C_t values reported as greater than 35 or as N/A (not detected) to 35. Any C_t value equal to 35 is considered a negative call.

3. When biological and/or technical replicates are performed, calculate the average C_t value of each gene (each well) across those replicate arrays for each treatment group.

4. Calculate the ΔC_t for each pathway-focused gene in each treatment group.
   ΔC_t (group 1) = average C_t – average of HK genes' C_t for group 1 array
   ΔC_t (group 2) = average C_t – average of HK genes' C_t for group 2 array

NOTE: *The expression level of the housekeeping genes chosen for normalization in the $\Delta\Delta C_t$ method must not be influenced by your experimental conditions. If one or more such genes have been previously identified by independent means and if the PCR Array reproduces those results, use the average of their C_t values in the equation above. If an appropriate housekeeping gene has not been previously identified, use the average C_t value of all five housekeeping genes, but only if the difference in the average values between the two groups to be compared is less than one (1) cycle. Otherwise, simply use zero (0) in the place of the* average of HK genes' C_t *for each group to be compared, and rely on the consistency in the quantity and quality of your original input total RNA across your groups to effectively normalize your results.*

5. Calculate the $\Delta\Delta C_t$ for each gene across two PCR Arrays (or groups).
   $\Delta\Delta C_t = \Delta C_t$ (group 2) - ΔC_t (group 1)
   Where group 1 is the control and group 2 is the experimental

6. Calculate the fold-change for each gene from group 1 to group 2 as 2^ ($-\Delta\Delta C_t$).

OPTIONAL: *If the fold-change is greater than 1, then the result may be reported as a fold up-regulation. If the fold-change is less than 1, then the negative inverse of the result may be reported as a fold down-regulation. The fold-change ratios may also be reported as is.*

NOTE: Detailed Mathematical Explanation of $\Delta\Delta C_t$ Data Analysis Method

Due to the inverse proportional relationship between threshold cycle and the original gene expression level, and the doubling of the amount of product of every cycle, the original expression level (L) for each gene of interest is expressed as:

$$L = 2^{-C_t}$$

To normalize the expression level of a gene of interest (GOI) to a housekeeping gene (HKG), the expression levels of the two genes are divided:

$$\frac{2^{-C_t\,(GOI)}}{2^{-C_t\,(HKG)}} = 2^{-[C_t\,(GOI) - C_t\,(HK)]} = 2^{-\Delta C_t}$$

To determine fold change in gene expression, the normalized expression of the GOI in the experimental sample is divided by the normalized expression of the same GOI in the control sample:

$$\frac{2^{-\Delta C_t\ expt}}{2^{-\Delta C_t\ control}} = 2^{-\Delta\Delta C_t}$$

Where $\Delta\Delta C_t$ is equal to ΔC_t expt – ΔC_t control

The complete calculation is as follows:

$$\frac{\dfrac{2^{-C_t\,(GOI)\ expt}}{2^{-C_t\,(HKG)\ expt}}}{\dfrac{2^{-C_t\,(GOI)\ control}}{2^{-C_t\,(HKG)\ control}}} = \frac{2^{-[C_t\,(GOI) - C_t\,(HK)]\ expt}}{2^{-[C_t\,(GOI) - C_t\,(HK)]\ control}} = \frac{2^{-\Delta C_t\ expt}}{2^{-\Delta C_t\ control}} = 2^{-\Delta\Delta C_t}$$

An Excel file downloadable from our web site automatically performs these calculations upon including a gene list and threshold cycle data from a real-time instrument. This Data Analysis template presents the results in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).

To download the template, visit the PCR Array home page at the following web address: http://www.superarray.com/PCRArrayPlate.php
Click the “PCR Array Data Analysis” link found in the lower “Product Support” section of the gray right-hand sidebar.

E. Alternate Protocol:
End-Point PCR Detection & Data Analysis Method:

Additional Materials Required:

1. RNA Isolation Kit: See real-time protocol for specific suggestions.
2. ReactionReady™ First Strand cDNA Synthesis Kit (Cat. No. C-01)
3. ReactionReady™ HotStart *"Sweet"* PCR Master Mix (Cat. No. PA-007)
4. SYBR® Green I: (Molecular Probes, Cat. No. S7563, 10,000X in DMSO)
5. Equipment: Standard thermal cycler and 96-well microplate fluorescence reader and appropriate optical 96-well plates designed for your reader

Protocol:

1. Perform Sample Preparation and Addition of Samples to PCR Array as described for real-time PCR.

2. Also, prepare a 5X solution of SYBR® Green.

Add 50 µl 10,000X concentrate SYBR® Green I to 5 ml DMSO. Mix well. Aliquot and store at -20 °C. On the day of the experiment, generate a 5X SYBR® Green I Solution. For each plate, mix 0.5 ml of the 100X SYBR® Green I Stock with 9.5 ml ddH_2O. Store on ice. Use only on the same day. Discard any remainder.

IMPORTANT: *PROTECT SYBR® Green I SOLUTIONS FROM LIGHT.*

NOTE: *To minimize variation, always freshly prepare and use the same batch of 5X SYBR Green for all arrays in the same experiment. Scale up the recipe as needed.*

3. Place one plate in thermal cycler. Enter and run the following program:

Cycles	Duration	Temperature
1	10 minutes[1]	95 °C
35	30 seconds	95 °C
	30 seconds	55 °C
	30 seconds	72 °C

[1]The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

4. Quantify the PCR yield from each well at the end of the PCR program:

   a. Transfer 3 µl of the PCR from each well to a fresh 96-well plate (not provided).
   b. Add 100 µl of 5X SYBR Green to each well in the new plates.
      Change the pipet tips each time to avoid cross-contaminating the reactions.
   c. Place the new plate in a 96-well microplate fluorescence reader.
   d. Read the fluorescence intensity with excitation at 485 nm and emission at 530 nm. The gain may need to be adjusted to place the whole plate in a detectable range.

NOTE: *Be sure to visually inspect the plate after the run for any signs of evaporation from any of the wells. If evaporation is observed, make a note of which wells so that you may qualify your data analysis appropriately.*

End-Point PCR Data Analysis:

1. Calculate the background-corrected relative expression level for each gene:

Background correction removes the contribution of non-specific signal intensity from each gene-specific fluorescence intensity value. Subtract the fluorescence reading in each well by the fluorescence reading of the No Template Control (NTC, Well H12). Change any resulting negative values to zero.

2. Determine which housekeeping gene(s) to use for normalization:

Normalization removes the influence of systematic variation between arrays so that numbers can be compared between them. Only use housekeeping genes (wells H1 through H5) that do not drastically change their values between the experimental conditions to be compared. For each PCR Array, average all of the background-corrected fluorescence intensity values for all useful housekeeping genes on that array.

3. Normalize the results:

Divide the background-corrected fluorescence intensity value of each gene of interest on each array by the average housekeeping gene value determined above for the same PCR Array.

4. Determine the fold-changes in relative gene expression:

Calculate the ratio between the background-corrected, housekeeping gene-normalized numbers for each gene across two different PCR Arrays (from two different samples).

OPTIONAL: *If the fold-change is greater than 1, then the result may be reported as a fold up-regulation. If the fold-change is less than 1, then the negative inverse of the result may be reported as a fold down-regulation. The fold-change ratios may also be reported as is.*

VI. Troubleshooting and Frequently Asked Questions

1. The "No Reverse Transcription" control (well H11, NRT) yields a real-time C_t value less than 35 cycles:

Contamination of the RNA with genomic DNA:
Be sure to include any DNase treatment steps in the recommended RNA isolation procedure or treat RNA separately with RNase-free DNase followed by re-purification using a spin-column based method (e.g., the Qiagen RNeasy Mini Kit).

If the genomic DNA contamination proves difficult to remove, fold-changes in gene expression may still be obtained. However, it will then be very important to validate any results for individual genes by a separate more rigorous real-time PCR analysis that includes a "minus RT" control.

2. The "No Template Control" (well H12, NTC) yields a real-time C_t value less than 35 cycles:

DNA contamination of other reagents, tips, and tubes:
See the Note on Preparing a Workspace Free of DNA Contamination at the beginning of the protocol in this User Manual.

3. Will pipetting error affect the PCR Array results?

The passive reference dyes in the PCR master mixes, such as ROX and Fluorescein, are used by the real-time PCR systems to normalize variation from well to well. Therefore, these systems tolerate volume variations caused by pipetting error and evaporation. The use of standard multi-channel pipettors will not affect the PCR Array results when passive reference dyes are used in PCR master mixes.

4. How can I prevent the evaporation of reaction volume from the wells?

Be sure to carefully and completely seal the PCR Array with the optical thin-wall 8-cap strips or the optical adhesive film before placing it into your thermal cycler.

5. How reliable are the results from the RT²*Profiler*™ PCR Array?

Assuming the use of good, consistent experimental technique, real-time PCR methods such as the PCR Array provide very reproducible results. To insure the reliability of your results and to reliably detect smaller fold changes in gene expression from the PCR Array, the performance of replicate determinations (duplicates or triplicates) is highly recommended. The Data Analysis Template available from our website for the PCR Array uses your replicate PCR Array data to calculate t-test p values and to generate a "Volcano Plot" illustrating the statistically significant fold-changes in gene expression.

6. How should I define the fluorescence intensity baseline for the calculation of the threshold cycle values?

Usually, the software that accompanies your real-time PCR system automatically calculates the average fluorescence background and the threshold cycle (C_t) values for each well in the array plate. Sometimes, the expression level of the 18S rRNA housekeeping gene is high enough to influence the automatic calculation of the baseline or threshold used to determine the C_t values from the rest of the wells. As a result, the software may report the 18S rRNA as not detectable and / or may artificially inflate the C_t values of the rest of the genes in the array. In these situations, manually setting the baseline or threshold position for the C_t value calculations will be necessary by following the recommended procedure below.

a. Select every well except the 18S rRNA well (H1) for data analysis.

b. Choose the first few cycles (for example, cycles 2 through 10) as those representing the average background fluorescence to help define the threshold value. Alternatively, you may define your own fluorescence threshold level.

c. Click the appropriate "recalculate" or "analysis" button according to the instrument manufacturer instructions, and the software will recalculate and re-plot all of the amplification curves.

d. Examine the semi-log amplification curves (cycle vs. Log (intensity)). Be sure that the threshold value crosses each of these curves within the lower two-thirds of exponential phase but above any background noise within the assay. The lower the threshold value, the more sensitive the results will be.

NOTE: *If you defined the threshold level yourself, you will also need to use the same threshold for all samples (arrays) that you plan to compare to one another. You may need to repeat this process a few times for each sample in order to meet these criteria.*

e. Export these C_t values.

f. Select only the 18S rRNA well (H1) for data analysis. In this case, define your own fluorescence threshold level close to that defined for the other wells above instead of relying on an automatic determination as described above. Repeat steps c through e, and export this C_t value separately.

g. Continue with the data analysis and calculation of fold changes in gene expression as described in this User Manual.

If you have additional questions, please check our website (www.superarray.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our Technical Support Representatives at 0800-2466651 or +49-40-853260-23,-27,-37.

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Appendix: Modified Protocol for Housekeeping Gene PCR Arrays

B. First Strand cDNA Synthesis

Perform a first strand cDNA synthesis reaction for each sample to be characterized on the array including one sample representing your experimental control.

C. Perform Real-Time PCR:

1. Sample Preparation:

   a. To characterize each sample in duplicate:

Mix the following components in a 1-ml tube or a multi-channel pipettor reservoir:	
2X SuperArray PCR master mix	337.5 µl
Diluted first strand cDNA synthesis reaction	27 µl
ddH_2O	310.5 µl
Total volume	675 µl

   Skip steps C.1.b., C.1.c., and C.1.d.

2. Adding samples to PCR Array:

NOTE: *Organize your sample loading onto the arrays very carefully making sure to characterize each sample in duplicate and to include a replicate of the control sample on each plate. For example, up to four samples can be characterized in duplicate on a single array or duplicate determinations may be made on two separate arrays for larger numbers of samples.*

Figure 4: Layout of the Housekeeping Genes PCR Arrays.

Housekeeping Genes

Samples	1	2	3	4	5	6	7	8	9	10	11	12
A	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
B	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
C	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
D	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
E	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
F	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12

D. Data Analysis by the $\Delta\Delta C_t$ Method

1. For each sample, average the duplicate determinations of the C_t values from each sample for each housekeeping gene.

2. For each housekeeping gene, calculate the ΔC_t, or, in other words, the difference between the gene's C_t value in each experimental sample and the same gene's C_t value in the control sample.

3. Choose the housekeeping genes with the smallest ΔC_t value across the samples of interest to normalize the results of your future RT-PCR experiments for input total RNA loading. More than one housekeeping gene may be chosen for your analyses. Simply monitor the expression of all of these housekeeping genes, and use their average C_t value as the normalization factor for each sample.

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RT²*Profiler*™ PCR Array

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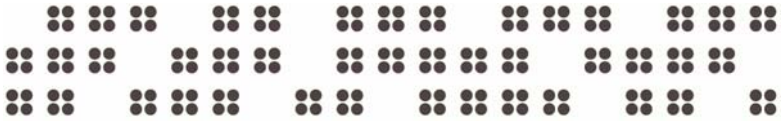
GENE EXPRESSION PROFILING IN A 96-WELL FORMAT

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)



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